

AMENDMENTS TO THE SPECIFICATION:

Please replace the paragraph beginning at page 4, line 1 with the following amended paragraph:

BRIEF SUMMARY OF THE ~~INVENTION~~ INVENTION

Please replace the paragraph beginning at page 5, line 19 with the following amended paragraph:

Figure 2 shows GAG-binding sequences of ~~[[four]]~~ six human genes: PF4, human platelet factor 4; IL8, human interleukin 8; AT III, human antithrombin III; ApoE, human apolipoprotein E; AAMP, human angio-associated migratory cell protein; human amphiregulin.

Please replace the paragraph beginning at page 19, line 17 with the following amended paragraph:

Clinically, a drug comprising aprotinin and an epithelial anchoring domain can be administered by aerosol inhalation to cover the entire respiratory tract to prevent and treat bronchopneumonia caused by influenza viruses, or any other virus, such as parainfluenza virus, that requires serine proteases in its life cycle. Alternatively, an aprotinin/~~epithelial~~ epithelial anchoring domain fusion protein can be administered as nasal spray to treat uncomplicated early stage influenza cases or other infections by respiratory viruses. In addition, an aprotinin/ ~~epithelial~~ epithelial anchoring domain fusion protein can be used as a prophylaxis for influenza or other viral infections before an infection occurs.

Please replace the paragraph beginning at page 21, line 3 with the following amended paragraph:

Preferred sialidases are the large bacterial sialidases that can degrade the receptor sialic acids Neu5Ac alpha(2,6)-Gal and Neu5Ac alpha(2,3)-Gal. For example, the bacterial sialidase enzymes from *Clostridium perfringens* (Genbank Accession Number X87369), *Actinomyces viscosus* (SEQ ID NO:12; Genbank Accession Number X62276), *Arthrobacter ureafaciens*, or *Micromonospora viridifaciens* (Genbank Accession Number D01045) can be used. Therapeutic domains of compounds of the present invention can comprise all or a portion of the amino acid sequence of a large bacterial sialidase or can comprise amino acid sequences that are substantially homologous to all or a portion of the amino acid sequence of a large bacterial sialidase. Other preferred sialidases are the human sialidases such as those encoded by the genes NEU2 (SEQ ID NO:8; Genbank Accession Number Y16535; Monti, E, Preti, Rossi, E., Ballabio, A and Borsani G. (1999) Genomics 57:137-143) and NEU4 (SEQ ID NO:9; Genbank Accession Number NM080741; Monti, E, Preti, A, Venerando, B and Borsani, G. (2002) *Neurochem Res* 27:646-663) (Figure 3). Therapeutic domains of

compounds of the present invention can comprise all or a portion of the amino acid sequences of a human sialidase or can comprise amino acid sequences that are substantially homologous to all or a portion of the amino acid sequences of a human sialidase. Preferably, where a therapeutic domain comprises a portion of the amino acid sequences of a naturally occurring sialidase, or sequences substantially homologous to a portion of the amino acid sequences of a naturally occurring sialidase, the portion comprises essentially the same activity as the human sialidase.

Please replace the paragraph beginning at page 23, line 15 with the following amended paragraph:

The pharmaceutically effective amount of a test compound required as a dose will depend on the route of administration, the type of animal or patient being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize. In practicing the methods of the present invention, the pharmaceutical compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized *in vivo*, preferably in a mammalian patient, preferably in a human, or *in vitro*. In employing them *in vivo*, the pharmaceutical compositions can be administered to the patient in a variety of ways, including topically, parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or ~~intraperitoneally~~ intraperitoneally, employing a variety of dosage forms. Such methods can also be used in testing the activity of test compounds *in vivo*.

Please replace the paragraph beginning at page 36, line 16 with the following amended paragraph:

1. Short-term culture of primary human epithelial cells. Conventional *in vitro* influenza virus infection is mostly carried out in MDCK cells with exogenous trypsin added to the culture medium. This is far from being physiological and is inappropriate for the work proposed here because trypsin is not the protease that activate influenza viruses *in vivo*. Very limited numbers of *in vitro* tissue culture models that are able to support the growth of influenza virus without an exogenous protease have been reported so far, those being primary cultures with primate cells of renal origin, cells lining the allantoic and ~~amniotic~~ amniotic cavities of embryonated eggs, fetal tracheal ring organ cultures and primary human adenoid epithelial cells (Endo Y, Carroll K N, Ikizler M R and Wright P F. (1996) *J Virol* 70:2055-2058). Among these, the latest work with primary human adenoid epithelial cells is the

closest mimic of human conditions. In this case, Endo *et. al.* (Endo Y, Carroll K N, Ikizler M R and Wright P F. (1996) *J Virol* 70:2055-2058) isolated epithelial cells from surgical samples of human adenoids, and cultured the epithelial cells on a collagen matrix (Vitrogen 100, Celtrix Laboratories, Palo Alto, Calif.) in Transwell inserts (Costar, Cambridge, Mass). Cells were maintained in 50% Ham's F12 and 50% Eagles minimal essential media with supplements of growth factors and trace elements. The cells reached confluency in 10 to 14 days, remaining largely as a monolayer but with discrete patches of ciliated cells, which maintained regular ciliary activity for 1 to 3 weeks after reaching confluency. In this system, influenza A virus grew to a titer of $10^{6.6}$ PFU/ml with a multiplicity of infection of 0.001 (Endo Y, Carroll K N, Ikizler M R and Wright P F. (1996) *J Virol* 70:2055-2058). Progressive cytopathogenic effects were also present during infection. The biggest drawback of this system is that it requires fresh human adenoid tissue.

Please replace the paragraph beginning at page 37, line 15 with the following amended paragraph:

2. Well-differentiated human airway epithelium (WD-HAE). In order to best mimic the in vivo condition of human airway, the model of well-differentiated human airway epithelium (WD-HAE) is used. WD-HAE is stratified epithelium that has all the differentiated cells of the normal human airway epithelium, including functional ciliated cells and mucus secreting cells. Therefore, in this model system influenza viruses are most likely to be activated by host proteases that are physiologically relevant. Although WD-HAE has been widely used to study respiratory viral infections, such as respiratory syncytial virus (RSV) (Zhang L, Peeples M E, Boucher R C, Collins P L and Pickles R J. (2002) *J Virol* 76:5654-5666) measles virus (Sinn P L, Williams G, Vongpunsawad S, Cattaneo R and McCray P B. (2002) *J Virol* 76:2403-2409, or human rhinovirus, it has not previously been used to study influenza viruses.

Please replace the paragraph beginning at page 40, line 2 with the following amended paragraph:

To demonstrate that aprotinin fusion proteins inhibit influenza viral infection by inhibiting cleavage of influenza HA protein, a human primary epithelial cell culture is infected with influenza virus at MOI of 1. Aprotinin fusion proteins are added to the culture either right before viral inoculation or immediately after the viral infection. At 6.5 hour post infection, the culture is incubated for 1 hour in MEM lacking cold methionine and containing ^{35}S -labeled methionine (Amersham) at a concentration of 100 microCi/ml (pulse). Thereafter, the cells are washed twice with MEM containing a 10-fold concentration of cold methionine

and incubated in MEM for additional 3 hours (chase). After labeling, cells are dissolved in radioimmunoprecipitation assay (RIPA) buffer, HA is precipitated by anti-serum against the particular strain of virus used for infection (anti-influenza sera can be obtained from ATCC and Center of Disease Control and Prevention), and the immunocomplex is then purified by protein G-Sepharose (Amersham). Samples are fractionated by SDS-PAGE followed by autoradiography. In samples untreated by aprotinin fusion proteins, HA1 and HA2 are expected to be the predominant HA species; while in aprotinin treated samples, HA0 is expected to be the major type of HA present.

Please replace the paragraph beginning at page 44, line 17 with the following amended paragraph:

Although the chances of inducing immune reactions using bacterial sialidases is very low because the proteins will be used topically in the upper respiratory tract and will not be absorbed ~~systemically~~ a systemically, a human enzyme would be more desirable for long-term use in human subjects.

Please replace the paragraph beginning at page 46, line 4 with the following amended paragraph:

Influenza viral strains are obtained from the ATCC and the repository at St. Jude Children's Research Hospital. ~~Viral~~ Viral stocks are grown on Madin-Darby canine kidney (MDCK) cells in minimal essential medium (MEM) supplemented with 0.3% bovine serum albumin and 0.5 micrograms of trypsin per ml. After incubating for 48 to 72 hours, the culture medium is ~~clarified~~ clarified by low speed centrifugation. Viral particles are pelleted by ultracentrifugation through a 25% sucrose cushion. Purified viruses are suspended in 50% glycerol-0.1 M Tris buffer (pH 7.3) and stored at -20°C. Viral titer is determined by plaque assay (Tobita, K, Sugiura, A, Enomoto, C and Furuyama, M. (1975) *Med Microbiol Immunol* 162: 9-14), or TCID₅₀, which is the dose of virus required to infect 50% of the MDCK cells.

Please replace the paragraph beginning at page 47, line 1 with the following amended paragraph:

Specifically, 6 ml of Chicken red blood cells (SPAFAS Inc., Norwich, Conn.) are diluted in two times the volume of PBS, centrifuge for 5 min at 500 × g and re-suspended in PBS of original volume. Sialidases are added to the chicken erythrocytes at various concentrations and allowed to incubate at room temperature for 30 min. The cells are then washed three times to remove sialidase proteins, and then are resuspended in PBS to 6 ml. Control cells are incubated with BSA and washed. Various strains of influenza virus, which recognize either Neu5Ac alpha(2,6)-Gal or Neu5Ac ~~alpha(2,3)-Gal~~ alpha(2,3)-Gal as the receptor as listed above, are prepared in microtiter plates as serial dilutions in PBS (100

microliters) of the original viral stocks. Sialidase-treated or control chicken red blood cell suspensions (100 microliters of the 0.5% solution prepared above) are added to each well at 4°C. The plates are read after 2 h. The lowest concentration of virus that causes the blood cell to agglutinate is defined as one hemagglutination unit. We will be looking for enzymes that effectively abolish hemagglutination by all viral strains.

Please replace the paragraph beginning at page 48, line 16 with the following amended paragraph:

We have selected six possible human GAG-binding sequences: PF4 (aa 47-70) (SEQ ID NO:2), IL-8 (aa 46-72) (SEQ ID NO:3), AT III (aa 118-151) (SEQ ID NO:4), ApoE (aa 132-165) (SEQ ID NO:5), ~~amphiregulin (aa 25-45)~~ human angio-associated migratory cell protein (AAMP) (aa 14-25) (SEQ ID NO:6), and ~~human angio-associated migratory cell protein (AAMP) (aa 14-25)~~ amphiregulin (aa 25-45) (SEQ ID NO:7) (Figure 2). These sequences generally bind to heparin with nanomolar-level affinities; however, their affinities may vary from one another by an order of magnitude (Table 1). Since it is not clear which anchoring domain will enable the most effective functioning of the sialidase, all four GAG-binding sequences are fused with the sialidase gene either on the N terminus or the C terminus via a generic linker sequence GGGGS as the following constructs:

Please replace the paragraph beginning at page 50, line 11 with the following amended paragraph:

Fusion proteins composed of a functional domain and an anchorage domain are designed for many more different applications. For example, a sialidase fusion protein as proposed here can also be used as a therapeutic/~~prophylactic~~ prophylactic agent against infections by other viruses and bacteria besides influenza viruses, because many other infectious microbes, such as paramyxoviruses (Wassilewa, L. (1977) *Arch Virol* 54:299-305), coronaviruses (Vlasak, R., Luytjes, W., Spaan, W. and Palese, P. (1988) *Proc Natl Acad Sci USA* 85:4526-4529), rotaviruses (Fukudome, K., Yoshie, O. and Konno, T. (1989) *Virology* 172:196-205) and ~~Pseudomonas aeruginosa~~ Pseudomonas aeruginosa (Ramphal, R. and Pyle, M. (1983) *Infect Immun* 41:339-44) etc, are also known to use sialic acid as cellular receptors. For example, aprotinin fused with a heparin-binding domain can make a fusion protein that be used to prevent/treat infection of other viruses besides influenza that require host serine proteases for activation, such as parainfluenza virus.